

Characterisation and expression of secretory phospholipase A₂ group IB during ontogeny of Atlantic cod (*Gadus morhua*)

Øystein Sæle*, Andreas Nordgreen†, Pål A. Olsvik and Kristin Hamre

National Institute of Nutrition and Seafood Research, PO Box 2029, Nordnes, 5817 Bergen, Norway

(Received 25 January 2010 – Revised 10 May 2010 – Accepted 13 May 2010 – First published online 14 September 2010)

Abstract

The pancreatic enzyme secretory phospholipase A₂ group IB (sPLA₂ IB) hydrolyses phospholipids at the sn-2 position, resulting in a NEFA and a lyso-phospholipid, which are then absorbed by the enterocytes. The sPLA₂ IB is a member of a family of nineteen enzymes sharing the same catalytic ability, of which nine are cytosolic and ten are secretory. Presently, there are no pharmacological tools to separate between the different secretory enzymes when measuring the enzymatic activity. Thus, it is important to support activity data with more precise techniques when isolation of intestinal content is not possible for analysis, as in the case of small teleost larvae, where the whole animal is sometimes analysed. In the present study, we characterise the sPLA₂ IB gene in Atlantic cod (*Gadus morhua*) and describe its ontogeny at the genetic and protein level and compare this to the total sPLA₂ activity level. A positive correlation was found between the expression of sPLA₂ IB mRNA and protein. Both remained stable and low during the larval stage followed by an increase from day 62 posthatch, coinciding with the development of the pyloric caeca. Meanwhile, total sPLA₂ enzyme activity in cod was stable and relatively high during the early stages when larvae were fed live prey, followed by a decrease in activity when the fish were weaned to a formulated diet. Thus, the expression of sPLA₂ IB mRNA and protein did not correlate with total sPLA₂ activity.

Key words: Phospholipid digestion: Pancreas: Western blot: Quantitative PCR: Enzyme activity

Phospholipids (PL) are an essential nutrient for fish larvae (see review by Coutteau *et al.*⁽¹⁾), and it has been demonstrated in fish larvae that increasing dietary PL levels can improve growth rates, increase survival^(2–4), reduce deformation⁽⁴⁾ and improve stress tolerance⁽⁵⁾. Generally, the dietary requirement of PL is high in fish larvae at the start of feeding and decreases as the fish approach the juvenile stage^(3,5,6). Although it is well known that the digestive system in altricial fish is still developing during the larval stage, it is still uncertain why there is such a high PL requirement during this period. Furthermore, it has been demonstrated that the digestive tract of sea bass (*Dicentrarchus labrax*) larvae matured faster and the larvae had increased growth, survival and decreased malformations when fed compound diets containing high levels of dietary PL from first feeding⁽⁷⁾. The same study also suggests that sea bass utilise dietary PL more efficiently than neutral lipids.

Digestion of PL is mainly catalysed by pancreatic phospholipase A₂ (phosphatide 2-acyl hydrolase, EC 3.1.1.4; PLA₂). The PLA₂ hydrolyses the fatty acid ester bond at

the sn-2 position of PL and produces a NEFA and a lysophospholipid⁽⁸⁾. Altogether, nineteen enzymes with PLA₂ activity have been described in mammals. Ten of these are of the secretory type and nine are cytosolic. One of the secretory PLA₂ (sPLA₂) is produced in the pancreas for the purpose of PL digestion: the sPLA₂ IB⁽⁹⁾. All the sPLA₂ are Ca²⁺ dependent and are recognised by a conserved Ca²⁺-binding loop as well as a conserved catabolic site. The distinctive feature separating the pancreatic form sPLA₂ IB involved in PL digestion from the other sPLA₂ is a domain called the pancreatic loop.

Unfortunately, there are no methods available today to distinguish between the enzymatic activities of the different PLA₂; at best, it is possible to discriminate between the cytosolic and secretory forms^(10,11). This presents a challenge when analysing small marine larvae where dissection of the digestive tract may be challenging, and therefore whole fish must be analysed. The analysis of PLA₂ activity in whole fish larvae will provide information of PL hydrolysis in all tissues involving a multitude of sPLA₂ enzymes, which will obscure information of the digestive capability

Abbreviations: dph, post hatch; GI-tract, gastrointestinal tract; PL, phospholipids; PLA₂, pancreatic phospholipase A₂; SL, standard length; sPLA₂, secretory phospholipase A₂.

* **Corresponding author:** Ø. Sæle, fax +47 55 90 52 99, email oyse@nifes.no

† Present address: NOFIMA Ingrediens, Kjerreidviken 16, 5141 Fyllingsdalen, Norway.

of specific phospholipases. It is therefore vital to accompany enzymatic activity analysis with more specific protein and mRNA quantification methods describing the enzyme in question.

Two low-molecular-weight sPLA₂ group IB have been characterised in the hepatopancreas of red sea bream (*Pagrus major*)⁽¹²⁾, and there is also a description of a PLA₂ in sea bass⁽⁴⁾. Little information on the ontogeny of PLA₂ activity in marine fish larvae is available, but Uematsu *et al.*⁽¹³⁾ did not find PLA₂ in acinar cells of the hepatopancreas in red sea bream before 13 d post hatch (dph). To shed light on the dynamics of sPLA₂ IB ontogeny in fish larvae, we have analysed the enzyme at the mRNA and protein level and compared this to total sPLA₂ activity. Due to multiplicity of enzymes in the PLA₂ family, we have emphasised the description of the distinctive parts of the pancreatic form of PLA₂. Furthermore, we discuss what different analytical tools may reveal about the production and activity of this one specific enzyme out of the multitude of other similar enzymes.

Material and methods

Biological parameters

Atlantic cod (*Gadus morhua*) larvae were sampled from three production tanks at the commercial hatchery Cod Culture Norway, in Rong, Norway, according to their fish holding routines, which are in accordance with the Animal Welfare Act of 12 December 1974, nr 73, §§22 and 30, amended 17 November 1998. Eggs were collected from natural spawning brood stock and incubated for 15 d at 7°C. Hatched embryos were transferred to three start feeding tanks (diameter: 3 m, depth: 1 m) and fed rotifers (*Brachionus plicatilis*) from 3 dph. Formulated diet (Gemma Micro, Skretting, Norway) was introduced to cod larvae at 18 dph and co-fed with rotifers until 30 dph. From 31 dph and onwards, larvae were fed solely on formulated diet. Fertilised eggs used for the production of larvae used in this work came from a brood stock tank containing fifty-five females and thirty-nine males. The batch of eggs may therefore come from multiple parents. The temperature in the three sampling tanks was $7.1 \pm 1^\circ\text{C}$ at 3 dph and was raised incrementally to 11°C by 17 dph.

The temperature was kept constant at $11.2 \pm 0.5^\circ\text{C}$ for the rest of the sampling period. Oxygen was measured and delivered continuously to larval tanks via an automated system with oxygen saturation ranging from 84 to 97% within tanks during the sampling period.

Biological sampling

Pooled samples of whole larvae and/or larvae gastrointestinal tract (GI-tract) were taken from the three tanks at every sampling point (Fig. 1) from 3 to 62 dph. At 74 dph, the three fish tanks were graded and approximately 35% of the smallest fish were removed. This was routinely done at the commercial facility where we sampled the larvae. The retained fish were sorted into a medium- and large-sized group. At 83 and 97 dph, fish were sampled from both the medium- and large-size group. Until 56 dph, the fish were collected using a 60 cm long glass pipette. From 63 dph, the fish were collected using a fish net. Fish were collected from approximately 40 cm below the surface and from the areas in the tank with the highest density of fish. The larvae were filtered using a plankton net screen which was patted dry from underneath with a paper towel to remove excess water from the larvae. All the larvae were killed by inserting a needle through the brain before dissection. The whole larvae were then collected in 1.5 ml Eppendorf tubes. The GI-tract was sampled by cutting off the head in a backward angle following the gills. This incision cut the oesophagus without spilling the stomach content. The intestinal tract was detached from the body cavity by an incision anterior to the anus opening, and then the GI-tract was carefully pulled out. The liver was carefully removed, but all other intestinal organs were included with the GI-tract samples. Whole larvae were sampled from 3 to 62 dph and the digestive tract was sampled from 34 to 98 dph (Fig. 1).

For RNA extraction, whole larvae or dissected GI-tract were pooled and homogenised with a pestle mortar in Eppendorf tubes containing 1 ml Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA). The samples were then put on dry ice for approximately 3–5 h before storing at -80°C until RNA extraction. For enzyme activity and Western blot, larvae and GI-tract samples were flash-frozen in liquid N₂ without any treatment. At every

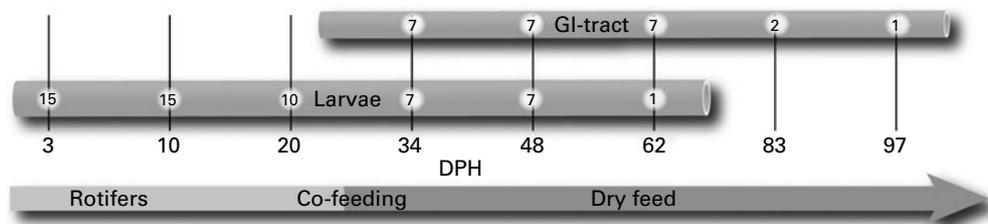


Fig. 1. Trial and sampling scheme. The arrow gives the type of diet during the sampling period, numbered circles indicate the number of pooled individuals in each sampling and the age of larvae at sampling is given in days post hatch (dph). GI, gastrointestinal tract.

sampling point, standard length (SL) (minimum ten fish/tank) was measured. Fish weight was measured from 56 dph (fifteen fish/tank). The fish were dried with paper and weighed individually. All the fish sampled for measuring length (SL) and weight were anaesthetised and killed with an overdose of metacaine (MS-222™; Norsk medisinaldepot AS, Bergen, Norway) dissolved in seawater. Dry weight was calculated according to Finn *et al.*⁽¹⁴⁾ based on SL. A size- and temperature-based growth model was used to evaluate the growth performance according to Folkvord⁽¹⁵⁾.

RNA extraction and quantitative PCR

The homogenised and stored in Trizol samples were thawed and thoroughly homogenised a second time, using a Precell 24 (Bertin Technologies, Montigny le Bretonneux, France). Total RNA was extracted from whole fish and GI-tract with Trizol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. The RNA-isolated samples were diluted in RNase-free double-distilled H₂O to 200 mg/μl and treated with DNA-free TM kit (Ambion, Austin, TX, USA) according to the manufacturer's descriptions. The quality of the RNA was assessed with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Optical density 260 nm/optical density 280 nm ratios for all total RNA samples ranged between 1.87 and 2.06.

Integrity of the RNA was controlled in twelve randomly chosen samples out of the thirty-six using the Agilent 2100 Bioanalyzer (Agilent Technologies) and the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA). RNA integrity numbers^(16,17) were between 8.9 and 10.

RT-PCR were run in duplicates on a ninety-six-well plate. For efficiency calculations, a twofold serial dilution of total RNA, mixed from all samples, ranging from 1000 to 31 ng/μl, was run in triplicates. All reactions were synthesised with a 500 ng total RNA input. Each plate included a no template control and RT-control (a reaction without RT enzyme). Plates were run on a GeneAmp PCR 9700 (Applied Biosystems, Foster City, CA, USA) using TaqMan Reverse Transcription Reagent containing Multiscribe RT (50 U/μl; 833.5 nkat/μl) (Applied Biosystems).

Reverse transcription was performed at 48°C for 60 min by using oligo dT primers (2.5 μM) for all the genes in 30 μl total volume. The final concentrations of the other chemicals in each RT reaction were MgCl₂ (5.5 mM), dNTP (500 mM of each), 10 × TaqMan RT buffer (1 ×), RNase inhibitor (0.4 U/μl) and Multiscribe RT (1.67 U/μl).

From each RT reaction, 2 μl of complementaryDNA and then 18 μl consisting of 7.6 μl double-distilled H₂O, 10 μl TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.2 μl of each primer (50 μM forward: GTCACGGTCTTGG-TGCTCTTG and reverse: GCCAAACTCATGACCATTGCT)

were pipetted in duplicate to 384-well plates. Pipetting was performed automatically by a robot (Biomek 3000; Beckman Coulter, Fullerton, CA, USA). Real-time PCR was run on the LightCycler® 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland), with a 5 min activation and denaturing step at 95°C, followed by forty cycles of a 15 s denaturing step at 95°C, a 60 s annealing step and a 30 s synthesis step at 72°C. The annealing temperature was 60°C for all primer pairs.

For the mean normalised expression calculations of the target genes, the geNorm VBA applet for Microsoft Excel 97 was used to calculate a normalisation factor based on three reference genes⁽¹⁸⁾. Ubiquitin, RPL 4 and RPL 37 were the selected reference genes based on Olsvik *et al.*⁽¹⁹⁾ and Sæle *et al.*⁽²⁰⁾, and the normalising factor calculated from these genes was used to calculate the mean normalised expression for each of the target genes.

Homogenisation

Whole larvae and dissected GI-tracts were homogenised in 4 × sample wet weight PBS with an Ultra – Turax® 20 000 (Upm, Janke u. Kunkel KG, Germany) followed by a second homogenisation in a ball mill (Retsch® MM301; Retsch GmbH, Haan, Germany), for 2 × 2 min (thirty shakes/s). Homogenates were centrifuged at 10 000 g for 15 min at 4°C and the supernatants were measured for total protein with the BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA), according to the manufacturer's instructions. The supernatant was used for analysis of enzyme activity and for Western blot analysis.

Enzyme activity

A sPLA₂ assay kit (product no. 765001; Cayman Chemical Company, Ann Arbor, MI, USA) was used for enzyme activity calculations. The assay uses the 1,2-dithio analogue of diheptanoyl phosphatidylcholine which serves as a substrate for most PLA₂ (for example, bee and cobra venoms, pancreatic fluid, etc.) with the exception of cytosolic PLA₂^(10,11). Upon hydrolysis of the thio-ester bond at the sn-2 position by PLA₂, free thiols are detected using 5,5-dithio-bis-(2-nitrobenzoic acid). Plates were read every 15 s for 30 min at 414 nm with a microreader (Labsystems iEMS Reader MF, Helsinki, Finland). Enzymatic activity was calculated accordingly:

$$\text{sPLA}_2 = \frac{\Delta A_{414/\text{min}}}{10.66 \text{ per mm}} \times \frac{0.225 \text{ ml}}{0.01 \text{ ml}} \times \text{sample dilution.}$$

$\Delta A_{414/\text{min}}$ is the change in absorbance/min, 10.66 per mm is the extinction coefficient for 5,5-dithio-bis-(2-nitrobenzoic acid) under the assay conditions.

Western blot

Polyclonal antibodies were produced in rabbits immunised with the PLA₂ peptide sequence: NPYTHGYHQTCDKSTK (Inbiolabs, Tallin, Estonia). Samples were resolved by denaturing SDS-PAGE using 12% polyacrylamide gels (BioRad, Hercules, CA, USA) and transferred to a polyvinylidene fluoride membrane (Millipore, Temecula, CA, USA). The membrane was blocked with 3% non-fat milk in TPBS (PBS, 0.1% Tween 20, pH 7.4) for 1 h, rinsed with TPBS and subsequently incubated with the primary antibody. Membranes were probed with primary anti-PLA₂ rabbit (dilution: 1:2000) overnight at 4°C. Membranes were then washed four times for 10 min each with TPBS, incubated for 1 h with the secondary anti-rabbit horse radish peroxidase conjugated (1:2000; Dako, Glostrup, Denmark) in blocking buffer. Membranes were then treated with ECL Plus Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ, USA) for 5 min and photographed on hyperfilm (GE Healthcare) in the dark. The negatives were scanned and the area of sPLA₂ IB protein bands was estimated using ImageJ software (<http://rsb.info.nih.gov>).

Open reading frame, alignments and statistics

Open reading frame sPLA₂ IB for Atlantic cod was generated with Baylor College of Medicine HGSC Search Launcher (<http://searchlauncher.bcm.tmc.edu/cgi-bin/seq-util/sixframe.pl>). Alignments were done in ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Potential differences were tested with one-way ANOVA. Newman–Keuls *post hoc* test was used to test for significant differences between group means in an ANOVA. Correlations were tested with multiple regression analysis. Differences and effects were considered significant at $P < 0.05$ for all tests and differences were annotated with different letters in figures. ANOVA and *post hoc* analyses were performed on Statistica 9.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

Larvae growth

Specific growth rate increased from 6.0% at 10 dph to peak at 11.9% at 34 dph when the SL was 7.75 mm. From 34 dph, there was a steady decrease in specific growth rate to 7.1% at 97 dph. To evaluate the growth performance of fish larvae used in the present study with potential growth in wild cod, a size- and temperature-based growth model by Folkvord⁽¹⁵⁾ was used:

$$\text{SGR} = 1.08 + (1.79T) - 0.074 \times T(\ln \text{DW}) - 0.0965 \times T(\ln \text{DW}) + 0.0112 \times T(\ln \text{DW})^3$$

T is temperature (°C) while DW is the dry weight (mg) of larvae at the first sampling point (3 dph). Dry weights

were calculated based on SL according to the following equations from Finn *et al.*⁽¹⁴⁾:

$$\text{Size range : } 0.031\text{--}0.175 \text{ mg DW : } \ln \text{SL} = \frac{(\ln(\text{DW} + 7.799))}{3.109}$$

$$\text{Size range : } 0.175\text{--}1.829 \text{ mg DW : } \ln \text{SL} = \frac{(\ln(\text{DW} + 9.657))}{4.129}$$

$$\text{Size range : } 1.829\text{--}409 \text{ mg DW : } \ln \text{SL} = \frac{(\ln(\text{DW} + 7.932))}{3.406}$$

There were an increase in the difference between the observed length growth (SL) and modelled growth throughout the ontogeny series. The increase in percent difference between the modelled and observed growth had a slope of 0.7 ($y = 0.6859x - 6.026$, $R^2 = 0.95$); consequently, the fish were 61.02 mm at 97 dph, whereas the model predicted a SL of 95.86 mm, a difference of 57% (Fig. 2).

Identification of the secretory pancreatic phospholipase A₂ IB gene

The expressed sequence tag (EST): EX726814 contained an open reading frame corresponding to a predicted amino acid sequence of matching sPLA₂ group IB (Fig. 3). Atlantic cod open reading frame similar to sPLA₂ group IB was aligned to sequences annotated to the PLA₂ superfamily of *D. labrax* (CAA10765), *P. major* (BAA23737) and *Fundulus heteroclitus* (AAU50527). Sequences of *Danio rerio* (AAI14294), *Sus scrofa* (NP_001004037), *Rattus norvegicus* (NP_113773) as well as *Homo sapiens* (NP_000919) annotated to sPLA₂ group IB were also aligned.

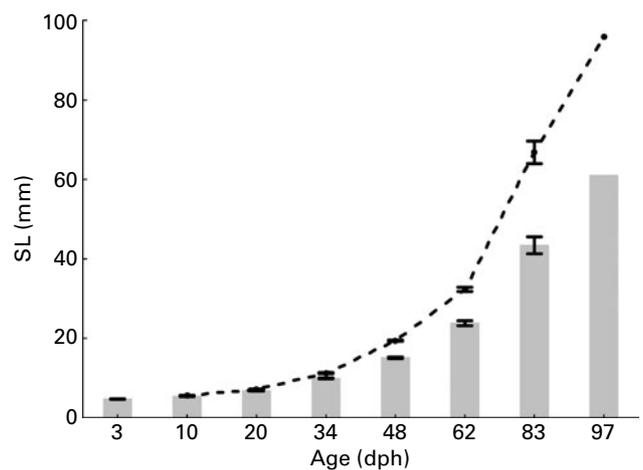


Fig. 2. Size at sampling. Standard length (SL) of Atlantic cod larvae at sampling points analysed (mean values with their standard errors). The curve gives the predicted growth based on the model: $\text{SGR} = 1.08 + (1.79T) - 0.074 \times T(\ln \text{DW}) - 0.0965 \times T(\ln \text{DW}) + 0.0112 \times T(\ln \text{DW})^3$ ⁽¹⁵⁾. The model is based on data from wild populations. There was a significant increase in SL from 20 days post hatch (dph) on (one-way ANOVA, $P < 0.05$). SGR, Specific growth rate; DW, dry weight.

```

ATGCTGACTATGGATGGTACTGTGGCAAGGGAGGCTCTGGAATACCTGTTG
A D 1 Y G W Y C G K G G S G I P V D
ATGCCCTCGACACATGCTGCCAAACTCATGACCATTGCTA CCACGCTGCAA
A L 2 D T C C Q T H D H C Y H A A K
AGGCAGATAAAGCGTGCATACCCTACCTGGATAACCCGTACACCCACGGCT
A D K A C I P Y L D N 3 P Y T H G Y
ACCACCAAACATGTGACCAAGAGCACCAAGACCCGTGAC CTGTCTCAGTACCA
H Q T C D K S T K T V T C L S T N
ATGATGCCCTGTCAGATGTTTATCTGCGAGTGTGACAAGAAGGCAGCCATGT
D A C Q M F I C E C D K K A A M C
GTTTTTCCCAGGCAAAATACATCCAAGCTCACGACCCTATAACCAGGATC
F S Q A K Y I Q A H D H Y N Q D L
TCTGTAGTAAATAAAATGAATACAACCAGGAGCTCTGTAGTAAATAAGATG
C S K * N E Y N Q E L C S K * D D
ATCAGGACCAAGAGCTCTGTTGTAATAAGATGACTAGGACCAGGAGCTCT
Q D Q E L C C K * D D * D Q E L C
GTAGTAAATAATGTGATAAGACCAGAAGCTCTGCAGGAAACAACCACACC
S K * C D K T R S S A G N N H T L
TCTGATCACCCCTCTATGGGCATACCTAATACATCCACTGGACACACACAAC
* S P S M G I P N T S T G H T Q Q
AGTGGGATGTTTAGGTATTCATTTATCAAACCTATTTGTCAATAAATAAATT
W D V * V F I Y Q T I C Q * N N

```

Fig. 3. Open reading frame based on EST (EX726814) from Atlantic cod. <http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html>. Sequences for the Ca-binding loop⁽²⁾, the catalytic domain of the enzyme⁽²⁾, the pancreatic loop⁽³⁾, epitope sequence used for antibody production (hatched box) and primers used for quantitative PCR (grey boxes).

To demonstrate that the previously listed sequences had the pancreatic loop unique to IB sPLA₂s, group V and X sPLA₂s were included in the alignment: *H. sapiens* (AAG43522) and *Mus musculus* (NP_001116426) from group V and *H. sapiens* (AAH69539), *M. musculus* (AAG43522) and *D. rerio* (NP_001002350) from group X. The Ca²⁺-binding loop (YGXXCGXGG) and the catalytic site (DXCCXXH) were conserved between all sequences aligned.

Assay verification

Atlantic cod sPLA₂ IB mRNA expression was assessed by RT-quantitative PCR in GI-tract, including pancreas, and also in the intestine, muscle, liver, gill, kidney and brain. The RT-PCR was run twenty-six and thirty-five cycles. After thirty-five cycles, all tissues except muscle produced a product when run on an ethidium bromide gel (data not shown). When the PCR was run twenty-six cycles, only GI-tract containing pancreatic tissue showed a band; all other tissues were blank (Fig. 4(a)). Ubiquitin was used as reference^(19,20). An increase from twenty-six to thirty-five PCR cycles gives a difference in amplification by the power of seven ($Y = x^7$).

Western blot of GI-tract homogenates showed only one band with the approximate size of 14 kDa (Fig. 4(b)). Enzymatic activity was tested in tissues of adult Atlantic cod. Activity was detected in liver and brain (mean 0.27 (SD 0.05) $\mu\text{mol}/\text{min}$ per ml) as well as in kidney and gill (mean 0.43 (SD 0.14) $\mu\text{mol}/\text{min}$ per ml), but no activity was detected in skeletal muscle (Fig. 4(c)).

Ontogeny of secretory pancreatic phospholipase A₂ group IB

The relative gene expression of PLA₂ (quantitative RT-PCR) in homogenates of whole larvae was very low at the start of feeding (3 dph) until 48 dph, ranging from 0.0004 (SD 0.0002) to 0.04 (SD 0.01), respectively. There was a significant increase at 62 dph to 0.17 (SD 0.11). In GI-tract homogenates, there was significant increases from 0.0003 (SD 0.0001) at 34 dph to 0.586 (SD 0.192) at 62 dph when it plateaued (Fig. 5(A)).

Protein expression was not detectable in whole fish homogenates until 62 dph. In GI-tract homogenates, there was an increase in protein expression from 48 to 62 dph and then again from 83 to 97 dph (Fig. 5(B)).

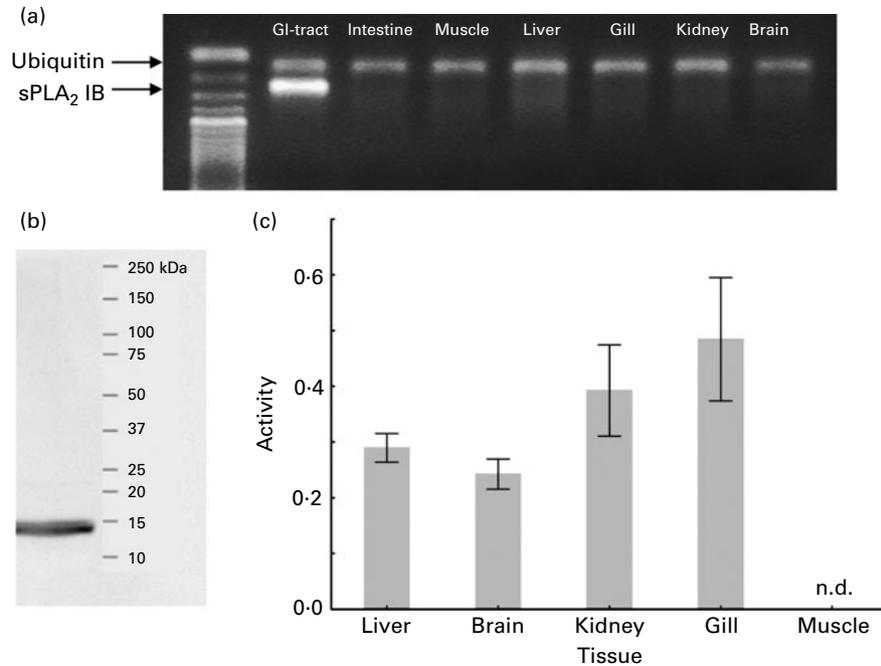


Fig. 4. (a) PCR of secretory phospholipase A₂ (sPLA₂) IB primers on mRNA from gastrointestinal tract (GI-tract) with attached pancreatic tissue, intestine, muscle, liver, gill, kidney and brain of adult cod. (b) Western blot with rabbit anti-sPLA₂ IB polyclonal antibody on homogenates of GI-tract. Only one band at 14 kDa was detected. (c) sPLA₂ activity in liver, brain, kidney, gill and muscle tissue. Activity could be detected in all tissues except muscle. n.d., Not detected.

There was a significant correlation between mRNA expression and protein expression both in larvae homogenates ($P < 0.0001$, $r^2 = 0.9$) and in GI-tract ($P = 0.004$, $r^2 = 0.5$) (Fig. 6).

Enzymatic activity was detectable already from first exogenous feeding. Homogenates of whole larvae had an sPLA₂ activity of $0.70 \mu\text{mol}/\text{min}$ per g from 3 to 20 dph. At 34 dph, the activity decreased to 0.36 (SD 0.04) $\mu\text{mol}/\text{min}$ per g. There was no increase in activity from 34 to 62 dph in larvae homogenates. The activity in GI-tract homogenates was stable from 34 to 97 dph, with an average activity of 1.11 (SD 0.18) $\mu\text{mol}/\text{min}$ per g (Fig. 5(C)). Enzymatic activity did not correlate with mRNA nor with protein expression.

Gastrointestinal-tract v. body secretory phospholipase A₂ specific activity

Average wet weights of the larvae at ages 34, 48 and 62 dph were 8.18, 34.9 and 146 mg, respectively. The dissected GI-tracts weighed 1.51, 2.21 and 9.49 mg at the same ages. Consequently, the GI-tract made up 18.5, 9.19 and 6.48% of the total body wet weight at 34, 48 and 62 dph, respectively. The average enzymatic activities at age 34, 48 and 62 dph were 0.0030, 0.014 and $0.064 \mu\text{mol}/\text{min}$ per fish, respectively. Average activities in dissected GI-tracts were 0.0019, 0.0043 and $0.0092 \mu\text{mol}/\text{min}$ per GI-tract. Hence, the activity of the GI-tract contributed with 63.2, 30.8 and 14.2% of the total body enzyme activity at 34, 48 and 62 dph, respectively (Fig. 7).

Discussion

The growth rate of cod larvae when fed natural zooplankton can be very high and is reported to range from 13 to 30%/d during the first 50 d^(14–21). Atlantic cod larvae fed live feed-like enriched rotifers (*B. plicatilis*) and *Artemia* and formulated diets generally have poor growth compared with those fed wild collected zooplankton⁽²²⁾. For example, cod larvae fed *Artemia* and formulated feed had a growth rate ranging from 5.1 to 10.7% between 22 and 64 dph^(23,24). Fish in the present study grew well compared with those in previous experiments evaluated using the same growth model⁽¹⁵⁾, by Busch *et al.*⁽²²⁾. The fish in the present study were 13.5 and 31.7% shorter than the modelled predictions at 9.9 mm SL (34 dph) and 23.8 mm SL (62 dph), respectively, while in another trial with a similar diet to that used in the present experiment, the larvae were 43.9 and 42.0% shorter than the model at a similar SL⁽²⁴⁾. We may therefore conclude that the ontogeny series of cod larvae analysed in the present study had good growth compared with other intensive rearing trials where larvae were start fed on rotifers and weaned at an early stage.

The family of sPLA₂ consists of the groups I, III, V and X, containing all together ten enzyme forms as described in mammals. The sPLA₂ IB belongs to a family of ten sPLA₂ that share a highly conserved region for a Ca²⁺-binding loop (XCGXGG) and a catalytic site (DXCCXXH)⁽⁹⁾. These sites are conserved between Atlantic cod and the evaluated fish, *D. labrax*, *P. major*, *D. rerio* and *F. heteroclitus* as well as in the mammals *S. scrofa*, *R. norvegicus*

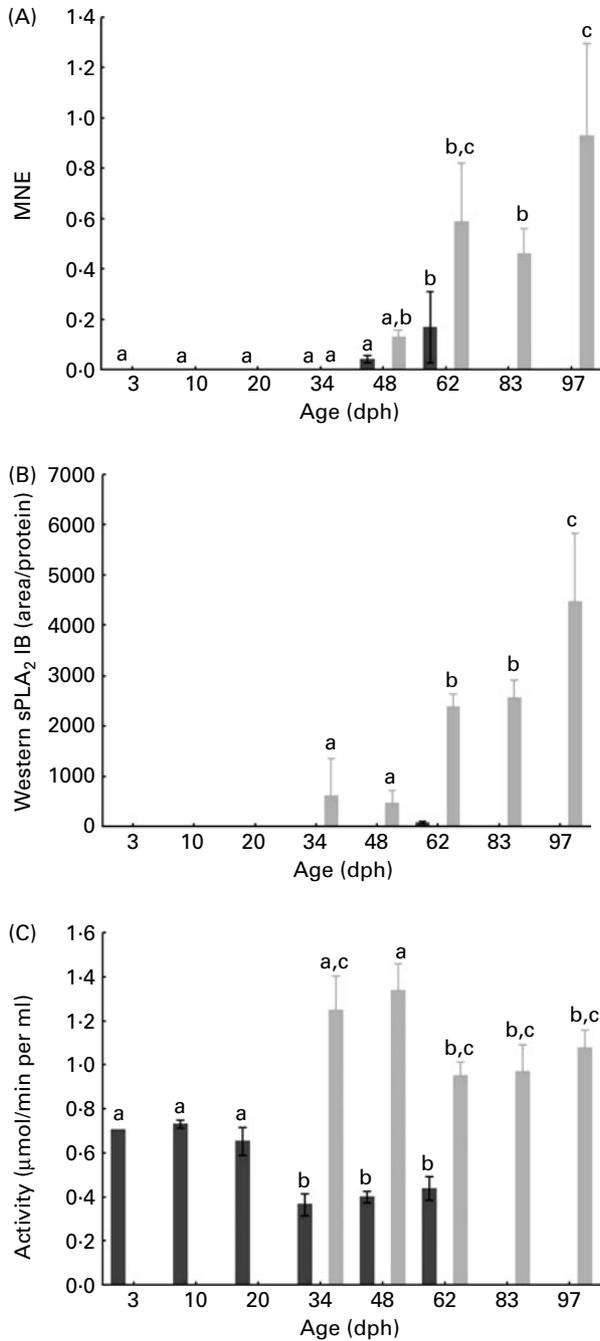


Fig. 5. Secretory phospholipase A₂ (sPLA₂) IB expression in whole Atlantic cod (■) and Atlantic cod gastrointestinal tracts (▒) at age 3 until 97 days post hatch (dph). (A) Mean normalised expression (MNE) of sPLA₂ IB mRNA (quantitative RT-PCR). (B) Relative quantity of protein (Western blot). (C) Enzyme activity. ^{a,b,c}Mean values with unlike superscript letters were significantly different (one-way ANOVA, $P < 0.05$); analysis of whole larvae homogenates and gastrointestinal tracts homogenates were not tested against each other.

and *H. sapiens*. However, in front of the Ca²⁺ loop, there is also a conserved region of the amino acids tyrosine (Y), glycine (G) and cysteine (C) between all listed species in Fig. 8, with the exception of Atlantic cod that has a tryptophan (W) in place of the C. The codon for C is UGU or UGC and for W it is UGG. Since C domains are much

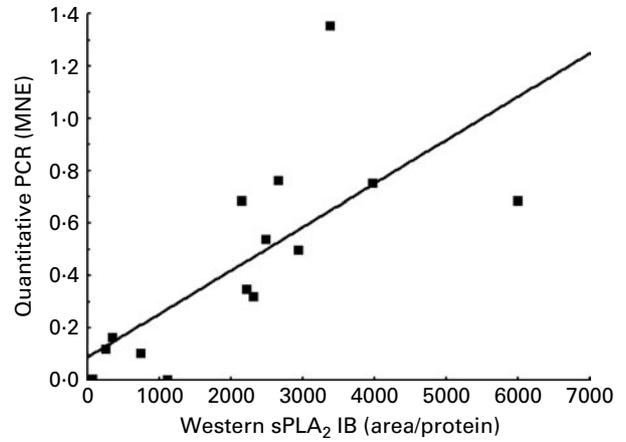


Fig. 6. Correlation between mRNA expression and protein expression of secretory phospholipase A₂ (sPLA₂) IB in gastrointestinal tract homogenates; $y = 0.086 + 0.0002 \times x$, $P = 0.0039$, $r^2 = 0.51$. MNE, mean normalised expression.

conserved⁽²⁵⁾, it is likely that the third G in the codon is a sequencing error and should in fact be a U or a C. With this exception, all C domains are conserved between the fishes listed in Fig. 8.

Distinctive for the pancreatic form sPLA₂ group IB involved in PL digestion is the pancreatic loop containing the domain PYTXX. This domain is conserved between all listed sPLA₂ with the exception of enzyme groups V and X and the *F. heteroclitus* sequence (Fig. 8). The sPLA₂ group V and X are not expressed in pancreatic tissue and are not involved in PL digestion⁽²⁶⁾. We may therefore conclude that the evaluated sequence in Atlantic cod most likely encodes for sPLA₂ IB. Further supporting this, the protein weight of sPLA₂ IB has previously been reported as 14 kDa^(9–29), which was the size of the protein picked up by our antibody. In addition, we also provide proof that the PLA₂ sequences from *D. labrax* and *P. major* probably should be annotated sPLA₂ IB.

The primers used in the present study may amplify PLA₂ isoforms present in other tissues than in the pancreatic tissue. Iijima *et al.*⁽²⁷⁾ characterised three isoforms of group I sPLA₂ in the gills of red sea bream, out of which one belonged to group IB. It is plausible that there are similar isoforms in Atlantic cod. The sPLA₂ IB has also been found in trace amounts in other organs, such as lung, kidney and spleen, in rat^(9–28). In the present study, mRNA expression of sPLA₂ IB in pancreas containing tissue was at least to the seventh power higher than in the liver, brain, kidney and gill. It is therefore likely that relative quantities of mRNA measured in GI-tract system as well as in whole larvae reflect the pancreatic production.

The enzyme activity assay used in the present study was specific for secretory enzyme forms, but cannot differentiate between different groups of sPLA₂. Analysing the homogenates of whole organisms and organ systems such as the GI-tract will therefore include the catalytic capability of PL sn-2 fatty acid hydrolysis of many tissues

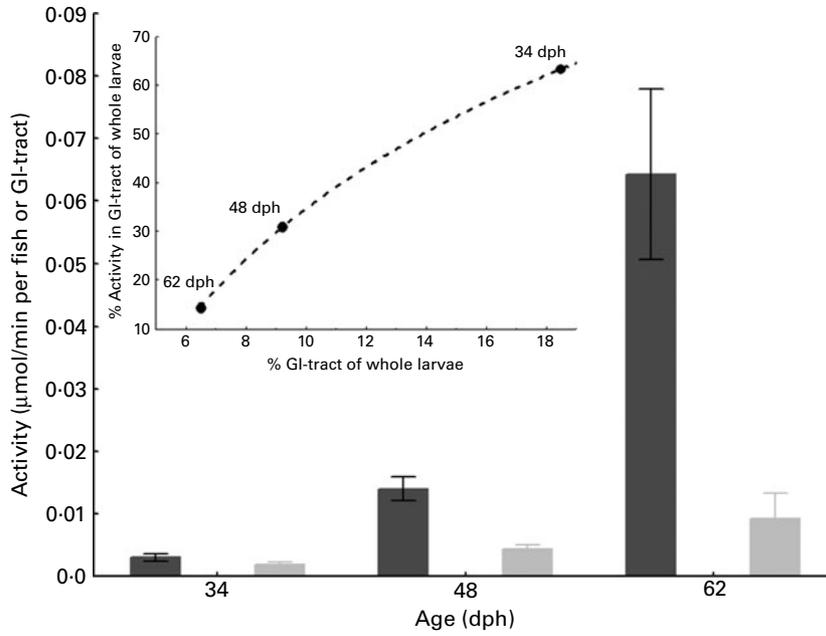


Fig. 7. The secretory phospholipase A₂ activity in whole Atlantic cod larvae (■) and their gastrointestinal (GI)-tract (▨). Inset shows correlation between percent size of GI-tract of whole larvae and percent activity in GI-tract to activity in whole larvae at ages 34, 48 and 62 d post hatch (dph).

and enzyme isoforms^(10,11). Iijima *et al.*⁽²⁷⁾ found that the PLA₂ activity in the gills of red sea bream was seventy times higher than in the pancreatic tissue. In the present study, the activity in gill tissue of juvenile cod was the

same as in kidney but less than half of the activity found in the GI-tract homogenate that included pancreatic tissue and gut content. Nevertheless, there is no doubt that enzyme activity data presented here and in other



Fig. 8. Alignment of the open reading frame amino acid sequences of phospholipase A₂ with the known pancreatic PLA₂ fish sequences: *Dicentrarchus labrax* (CAA10765), *Pagrus major* (BAA23737), *Fundulus heteroclitus* (AAU50527), *Danio rerio* (XP_700448) and the secretory PL A₂ (sPLA₂) IB mammalian sequences: *Sus scrofa* (NP_001004037), *H. sapiens* (NP_000919) and *R. norvegicus* (NP_113773). For visualisation of characteristics of the pancreatic form of sPLA₂, sequences of the group V sPLA₂: *M. musculus* (EDL13282) and *H. sapiens* (AAG43522) and the group X sPLA₂: *M. musculus* (NP_001116426), *Homo sapiens* (AAH69539) and *D. rerio* (NP_001002350) were included in the alignment. Accession numbers are given in parenthesis. Sequences for the Ca-binding loop (1), the catalytic domain of the enzyme (2), the pancreatic loop (3). * Indicates conserved amino acid residues identical to those of Atlantic cod, - indicates amino acid residues absent in the sPLA₂ groups V and X.

papers⁽³⁰⁾ represent the activity of a cocktail of PLA₂ types from various tissues. There have been attempts to minimise this problem. For example, Cahu *et al.*⁽⁷⁾ divided the larvae in segments, but the pancreas segment probably still included the parts of kidney and liver, the important contributors to PLA₂ activity.

The mRNA expression of sPLA₂ IB in whole fish was stable but low at the start of feeding, but increased from 48 dph and 15.1 mm SL, and was higher again at 62 dph and 23.8 mm SL. Uematsu *et al.*⁽¹³⁾ did not find a sPLA₂ IB immunohistochemical signal until 13 dph in pancreatic acinar cells from red sea bream, which is 11–9 d post first exogenous feeding⁽³¹⁾. The intensity of staining then increased steadily during development. In the present study, sPLA₂ IB Western blots of homogenates from larvae did not pick up any signal until 62 dph. This together with the findings of Uematsu *et al.*⁽¹³⁾ demonstrates that the quantity of sPLA₂ IB is under the detection limit of these assays and therefore quite low during the early larval stages of both red sea bream and Atlantic cod. However, Ozkizilcik *et al.*⁽³⁰⁾ measured PLA₂ activity on whole eggs and larvae of striped bass (*Morone saxatilis*) and found a steady increase in activity from fertilisation until the juvenile stage. As emphasised by the authors, activity assays register all types of PLA₂ activity and are unsuitable for assessment of isoform-specific activity during development. This is further emphasised by the elevated PLA₂ activities in starved larvae when compared with fed larvae⁽³⁰⁾. An important difference between the present data and those of Ozkizilcik *et al.*⁽³⁰⁾ is that while they analysed larvae with empty guts, we analysed larvae with filled guts. The latter would then include any sPLA₂ activity of ingested rotifers in addition to increased enzymatic activity of the larvae. This is visualised by the significant drop in whole larvae sPLA₂ activity after 20 dph when weaning of larvae onto the formulated diet began (Fig. 5(C)). This drop is probably due to the absence of rotifer enzyme activity and not the decreased production of sPLA₂ since it is only seen in activity and not in mRNA status (Fig. 5). According to Infante & Cahu⁽³²⁾, the levels of PL in the diet regulate sPLA₂ mRNA in *D. labrax* larvae. However, no such regulation was apparent in cod from the present trial. The formulated diet contained 14–15% total lipid of DM of which 80–86% was PL (Gemma micro), whereas the rotifers fed had a total lipid content of 8.5 (SD 1.8)% of DM of which 37 (SD 3)% is PL⁽³³⁾. Hence, when the larvae are weaned, they change from a low PL diet of 3.15% to a high PL diet of 12% of dry weight. Instead of up-regulating the sPLA₂ IB production and sPLA₂ activity to accommodate for the increase in dietary PL, the enzyme production is unaffected and the enzymatic activity is decreased.

There was a linear correlation between the mRNA expression and the protein expression in GI-tract homogenates, with a coefficient of determination of 0.51 (Fig. 6). The mRNA of sPLA₂ IB is only found in pancreatic

acinar cells, whereas the protein would be present in the intestinal lumen as well in the pancreas⁽⁹⁾. The remaining 49% of the variation between the quantity of mRNA and protein can therefore be explained by post-transcriptional regulations of protein synthesis and by enzyme present in the gut lumen.

In Atlantic cod, we found a close relationship between the ratio of GI-tract to whole larvae wet weight and the ratio of GI-tract to total larvae enzyme activity. In turbot larvae, 61% of the neutral lipolytic activity came from the gut at 12 dph decreasing to 52% at 24 dph⁽³⁴⁾. This coincides with the trend shown in cod and is probably correlated with the decreasing proportion of the GI-tract to whole body mass during ontogeny.

A question that can be asked based on the present results is: what does the ontogeny of the digestive enzyme expression and activity tell us about the digestive capabilities of the fish larvae? The answer is that it appears to tell only part of the story, i.e. when the adult form of digestion develops. Fish larvae require PL-rich diets^(6,35) and it has been demonstrated that intact PL is absorbed in intestinal epithelium in larval zebrafish^(36,37). We propose that in the early larval stages, the endogenous production of sPLA₂ IB is low and exogenous enzyme contribution from live prey is important for PL digestion in addition to the ability of the larvae to absorb intact PL. During ontogeny, these mechanisms may disappear and/or become less important in parallel with an increase in the endogenous enzyme production as the fish acquire the adult digestion form.

Acknowledgements

We thank Line Muklebust for valuable help with Western blots and Tina Constance Rosvold for valuable help during the tedious sampling and dissection of GI-tracts. We also thank Samuel James Penglase for painstaking correction of the manuscript and for the valuable inputs. We thank Marine Harvest Cod AS. This work is a part of the project financed by the Research Council of Norway: ontogeny of lipid digestion and the effects of feeding pre-digested lipids to Atlantic cod (*G. morhua*) larvae 179016/S40. There are no conflicts of interest. A. N. was responsible for sampling and RNA extraction. P. A. O. identified sPLA₂ IB in Atlantic cod and designed quantitative PCR primers. Ø. S. performed the mRNA, protein, enzyme and bioinformatics analysis. K. H. planned the study and was the project leader, and together with Ø. S., he is the major author of the manuscript. All the authors contributed to writing the manuscript.

References

1. Coutteau P, Geurden I, Camara MR, *et al.* (1997) Review on the dietary effects of phospholipids in fish and crustacean larviculture. *Aquaculture* **155**, 149–164.

2. Radunzneto J, Corraze G, Charlon N, *et al.* (1994) Lipid supplementation of casein-based purified diets for carp (*Cyprinus carpio* L.) larvae. *Aquaculture* **128**, 153–161.
3. Geurden I, Coutteau P & Sorgeloos P (1995) Dietary phospholipids for European sea bass (*Dicentrarchus labrax* L.) during first on-growing. *Larvi '95 – Fish and Shellfish Symposium, Gent, Belgium September 3–7, EAS Special Publication*, vol. 24, pp. 175–178 [P Lavens and E Jaspers, *et al.*, editors].
4. Cahu CL, Infante JL & Barbosa V (2003) Effect of dietary phospholipid level and phospholipid: neutral lipid value on the development of sea bass (*Dicentrarchus labrax*) larvae fed a compound diet. *Br J Nutr* **90**, 21–28.
5. Kanazawa A (1997) Effects of docosahexaenoic acid and phospholipids on stress tolerance of fish. *Aquaculture* **155**, 129–134.
6. Cahu CL, Gisbert E, Villeneuve LAN, *et al.* (2009) Influence of dietary phospholipids on early ontogenesis of fish. *Aquac Res* **40**, 989–999.
7. Cahu C, Infante JZ & Takeuchi T (2003) Nutritional components affecting skeletal development in fish larvae. *Aquaculture* **227**, 245–258.
8. Izquierdo MS, Socorro J, Arantzamendi L, *et al.* (2000) Recent advances in lipid nutrition in fish larvae. *Fish Physiol Biochem* **22**, 97–107.
9. Murakami M & Kudo I (2002) Phospholipase A₂. *J Biochem* **101**, 285–292.
10. Hendrickson HS, Hendrickson EK & Dybvig RH (1983) Chiral synthesis of a dithiolester analog of phosphatidylcholine as a substrate for the assay of phospholipase-A₂. *J Lipid Res* **24**, 1532–1537.
11. Reynolds LJ, Hughes LL & Dennis EA (1992) Analysis of human synovial-fluid phospholipase-A₂ on short chain phosphatidylcholine-mixed micelles – development of a spectrophotometric assay suitable for a microtiterplate reader. *Anal Biochem* **204**, 190–197.
12. Iijima N, Fujikawa Y, Tateishi Y, *et al.* (2001) Cloning and expression of group IB phospholipase A(2) isoforms in the red sea bream *Pagrus major*. *Lipids* **36**, 499–506.
13. Uematsu K, Kitano M, Morita M, *et al.* (1992) Presence and ontogeny of intestinal and pancreatic phospholipase A₂-like proteins in the red sea bream, *Pagrus major* – an immunocytochemical study. *Fish Physiol Biochem* **9**, 427–438.
14. Finn RN, Ronnestad I, van der Meer T, *et al.* (2002) Fuel and metabolic scaling during the early life stages of Atlantic cod *Gadus morhua*. *Marine Ecology-Progress Series* **243**, 217–234.
15. Folkvord A (2005) Comparison of size-at-age of larval Atlantic cod (*Gadus morhua*) from different populations based on size- and temperature-dependent growth models. *Can J Fish Aquat Sci* **62**, 1037–1052.
16. Mueller O, Hahnenberger K, Dittmann M, *et al.* (2000) A microfluidic system for high-speed reproducible DNA sizing and quantitation. *Electrophoresis* **21**, 128–134.
17. Imbeaud S, Graudens E, Boulanger V, *et al.* (2005) Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucleic Acids Res* **33**, e56.
18. Vandesompele J, De Preter K, Pattyn F, *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, 0034-0031–0034-0011.
19. Olsvik PA, Softeland L & Lie KK (2008) Selection of reference genes for qRT-PCR studies of wild populations of Atlantic cod *Gadus morhua*. *BMC Res Notes* **16**, 1–47.
20. Saele O, Nordgreen A, Hamre K, *et al.* (2009) Evaluation of candidate reference genes in Q-PCR studies of Atlantic cod (*Gadus morhua*) ontogeny, with emphasis on the gastrointestinal tract. *Comp Biochem Physiol Biochem Mol Biol* **152**, 94–101.
21. Otterlei E, Nyhammer G, Folkvord A, *et al.* (1999) Temperature- and size-dependent growth of larval and early juvenile Atlantic cod (*Gadus morhua*): a comparative study of Norwegian coastal cod and northeast Arctic cod. *Can J Fish Aquat Sci* **56**, 2099–2111.
22. Busch KET, Falk-Petersen IB, Peruzzi S, *et al.* (2009) Natural zooplankton as larval feed in intensive rearing systems for juvenile production of Atlantic cod (*Gadus morhua* L.). *Aquac Res* (epublication ahead of print version 28 December 2009).
23. MacQueen Leifson R, Homme JM, Lie O, *et al.* (2003) Three different lipid sources in formulated start-feeds for turbot (*Scophthalmus maximus* L.) larvae – effect on growth and mitochondrial alteration in enterocytes. *Aquac Nutr* **9**, 33–42.
24. Callan C, Jordaan A & Kling LJ (2003) Reducing Artemia use in the culture of Atlantic cod (*Gadus morhua*). *Aquaculture* **219**, 858–895.
25. Sevier CS & Kaiser CA (2002) Formation and transfer of disulphide bonds in living cells. *Nat Rev Mol Cell Biol* **3**, 836–847.
26. Triggiani M, Granata F, Giannattasio G, *et al.* (2005) Secretory phospholipases A(2) in inflammatory and allergic diseases: not just enzymes. *J Allergy Clin Immunol* **116**, 1000–1006.
27. Iijima N, Uchiyama S, Fujikawa Y, *et al.* (2000) Purification, characterization, and molecular cloning of group I phospholipases A(2) from the gills of the red sea bream, *Pagrus major*. *Lipids* **35**, 1359–1370.
28. Tojo H, Ono T, Kuramitsu S, *et al.* (1988) A phospholipase-A₂ in the supernatant fraction of rat spleen – its similarity to rat pancreatic phospholipase-A₂. *J Biol Chem* **263**, 5724–5731.
29. Schaloske RH & Dennis EA (2006) The phospholipase A(2) superfamily and its group numbering system. *Biochim Biophys Acta* **1761**, 1246–1259.
30. Ozkizilcik S, Chu FLE & Place AR (1996) Ontogenetic changes of lipolytic enzymes in striped bass (*Morone saxatilis*). *Comp Biochem Physiol Biochem Mol Biol* **113**, 631–637.
31. Nguyen VT, Satoh S, Haga Y, *et al.* (2008) Effect of zinc and manganese supplementation in *Artemia* on growth and vertebral deformity in red sea bream (*Pagrus major*) larvae. *Aquaculture* **285**, 184–192.
32. Infante JL & Cahu CL (1999) High dietary lipid levels enhance digestive tract maturation and improve *Dicentrarchus labrax* larval development. *J Nutr* **129**, 1195–1200.
33. Hamre K, Mollan TA, Saele O, *et al.* (2008) Rotifers enriched with iodine and selenium increase survival in Atlantic cod (*Gadus morhua*) larvae. *Aquaculture* **284**, 190–195.
34. Hoehne-Reitan K, Kjørsvik E & Reitan KI (2003) Lipolytic activities in developing turbot larvae as influenced by diet. *Aquac Int* **11**, 477–489.
35. Tocher DR, Bendiksen EA, Campbell PJ, *et al.* (2008) The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture* **280**, 21–34.
36. Carten JD & Farber SA (2009) A new model system swims into focus: using the zebrafish to visualize intestinal lipid metabolism *in vivo*. *Clin Lipidol* **4**, 501–515.
37. Farber SA, Pack M, Ho SY, *et al.* (2001) Genetic analysis of digestive physiology using fluorescent phospholipid reporters. *Science* **292**, 1385–1388.